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TOWNSEND and TOWNSEND and CREW

By R. M. Vallee

OIPE JC19 MAY 24 1999 PATENT & TRADEMARK OFFICE

PATENT

Attorney Docket No. 16994-003123US

#25

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Herman A. DeBoer et al.

Application No. 08/461,333

Filed June 5, 1995

For PRODUCTION OF RECOMBINANT POLYPEPTIDES BY BOVINE SPECIES AND TRANSGENIC METHODS

Examiner: D. Crouch

Art Unit: 1819

DECLARATION OF FRANK PIEPER under §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Frank Pieper, Ph.D., a co-inventor of the above-captioned application, state as follows.

1. My present position is Director, Technology Development, at Pharming B.V., the assignee of the above-captioned application. I joined Pharming in 1989. I have extensive expertise in molecular and cell biology and have served Pharming in various capacities (Scientist, Director Research, Director Research and Development). I have been instrumental in developing and managing various aspects of Pharming's transgenic and gene expression technology. Before joining Pharming, I conducted research at the Universities of Leuven, Belgium and Nijmegen, The Netherlands (1985-88) in the area of targeted expression of heterologous proteins in various systems, including transgenic animals.

2. I have reviewed the above-captioned application, and the office action mailed May 19, 1997. The application claims transgenic bovines producing a detectable amount of a polypeptide of interest in their milk. The transgene in such a bovine comprises a DNA sequence encoding the polypeptide of interest in operable association with regulatory sequences from a mammary-gland specific gene, and a DNA sequence encoding a signal sequence functional in bovine mammary secretory cells. I understand the Examiner takes the position that it was unpredictable whether a transgene as described above would be capable of directing the expression of detectable amounts of a polypeptide in milk. In this declaration, I discuss examples in which transgenic bovines having transgenes of the composition described above do direct expression of several different polypeptides in the milk of transgenic cattle.

3. Example 15 of the application describes the production of a transgenic bovine having a construct including a cDNA sequence encoding human lactoferrin linked to 5' and 3' casein flanking sequences. The flanking sequences include a casein promoter, enhancer and signal sequence. We have bred the transgenic bovine described in the Example to generate eight female transgenic offspring (F1 generation). Five of these animals were made pregnant by inseminating them with sperm from a nontransgenic bull. After delivery (resulting in three transgenic calves of the F2 generation, one female and two male), the five animals from the F1 generation were milked. The level of human lactoferrin in milk was determined by radioimmunoassay using an antibody specific for human lactoferrin. The concentration of human lactoferrin in the milk was 0.2-9 mg/L. By contrast milk from a nontransgenic cow showed undetectable levels of human lactoferrin.

4. Example 19 of the above-captioned application describes the construction of a transgene for expression of human lactoferrin the milk of transgenic bovines. The transgene designated BhLFgen37 has a 6.2 kb 5' αs1 casein fragment

containing a casein promoter, enhancer, fused to a genomic human lactoferrin coding and signal sequence. The transgene has been introduced into bovine embryos and transgenic bovines were generated by the *in vitro* procedure described in Examples 6 and 15 of the application.

Around day 69-90 of pregnancy, transvaginal amniocentesis was performed in order to determine sex and transgenesis. Non-transgenic pregnancies were terminated. Following these procedures, we identified five transgenic pregnancies. Southern blotting analysis of DNA obtained from multiple tissues of the calves after birth confirmed transgenesis. Four of these animals contained one or more intact copies of the transgene, one animal contained only part of the transgene. Three of the animals harboring intact transgenes were bred and shown to transmit the transgene both *in vitro* (cultured embryos) and *in vivo* (calves).

Lactation of female calf Ike was induced with hormones at the age of 8 months. Ike was milked twice a day and the volume of milk collected gradually increased to remain stable at about 1.5 liter daily from day 30 until day 60 when milking was finished. Human lactoferrin levels in milk were measured by radioimmunoassay using an antibody specific to human lactoferrin. The concentration of human lactoferrin increased from an initial level of 0.2 g/L to about 1 g/L during the first week of lactation. On day 20, hLF slightly decreased to 0.8 g/l, and remained at that level throughout the milk collection period. Control tests on milk from nontransgenic cows showed undetectable human lactoferrin.

5. Another example of a transgenic bovine expressing a foreign protein in milk has been described by Hyttinen et al., *Bio/Technology* 12, 606-608 (1994).[1] Briefly, this transgenic bovine has a transgene containing an αs1-casein fragment containing upstream regulatory sequences, a signal sequence, a genomic sequence encoding the protein erythropoietin and a 3'

[1] The transgenic bovine described by Hyttinen ~~and its progeny~~ are now in the custody of Pharming B.V., the assignee of the above-captioned application. F.P. 8/22/97

flanking asl sequence. The transgene was introduced into transgenic bovines by the *in vitro* procedure described in the present application and a female transgenic bovine was produced. Lactation of this female calf has been induced by a short hormonal treatment as above. The animal responded very well by starting to produce significant amounts of milk. The levels of EPO were measured by an activity assay that measures EPO-induced growth stimulation of a pluripotent cell line. Milk from the transgenic bovine contained levels of around 20-25 mg/L human EPO whereas levels of EPO in control milk from non-transgenic calves were below the limits of detections (i.e., less than 0.005 mg/L). The observed levels in the milk of transgenic bovines were, in fact, higher than observed in transgenic mice bearing the same construct.

6. A still further example of a transgenic bovine expressing a foreign protein in milk has been described by Dr. Colman of PPL Therapeutics on February 7, 1997 at an IBC-meeting in Florida, which I attended (copies of overheads from the meeting are attached). Dr. Colman reports generation of transgenic bovines carrying a mammary gland-specific transgene encoding human alpha-lactalbumin. The strategy used by Dr. Colman for generating transgenic bovines is the same as described in the present application. That is, ova were obtained from slaughterhouse ovaries, matured and fertilized *in vitro*, microinjected with transgene, further cultured and introduced into recipient cows (see attached overheads from meeting). Several males and one female transgenic calf incorporating an alpha-lactalbumin transgene were reported by Dr. Colman at the IBC meeting. The female transgenic calf was induced to lactate by the same hormonal treatment as described above. In this case, the animal was reported to respond well to the treatment and to produce milk. This milk was reported to contain human alpha-lactalbumin at about 2.4 g/L.

7. In summary, the above examples show that independent lines of bovines incorporating transgenes described

in the present application express detectable levels of foreign protein in their milk. The above examples also show that two other foreign proteins have been expressed in transgenic bovines produced according to the teachings of the present application. In each case, expression was directed by a transgene comprising a protein-coding sequence and mammary-gland specific regulatory sequences. In each case, the transgene was introduced into transgenic bovines using the *in vitro* procedure described in the present application. The strategy underlying the transgene design, construction and incorporation into transgenic bovines is independent of the nature of the protein to be expressed. Thus, I would expect that many proteins can be expressed at detectable levels in the milk of transgenic bovines by the same means. For these reasons, I conclude that transgenic bovines having transgenes as described and claimed in the application could have been generated and used to produce many different kinds of protein in their milk according to the teaching of the application (and the predecessors from which it derives priority) and common knowledge in the art at the priority date of the application (Dec. 1989).

8. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 8/22/1997

Frank Pieper